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Intramembrane translocation and posttranslational palmitoylation of the chloroplast 32-kDa herbicide-binding protein

(*Spirodela oligorrhiza*/photosystem II/plastid proteins/protein translocation/acylation)

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ABSTRACT **The 32-kDa herbicide-binding protein, a component of photosystem II, is synthesized as a membrane-associated 33.5-kDa precursor within the chloroplast. We show that membrane attachment of the precursor and processing to the 32-kDa form occur in the unstacked stromal lamellae. Once processed, the 32-kDa protein translocates, within the thylakoids, to the topologically distinct stacked granal lamellae. Posttranslational palmitoylation of the processed 32-kDa protein is also shown to occur. This modification takes place in a membrane-protected domain and is mainly confined to the protein assembled in the granal lamellae, where functional photosystem II centers are concentrated.**

The internal membranes (thylakoids) of the chloroplast constitute the sites where transformation of light energy into chemical energy is catalyzed by multisubunit complexes composed of pigments, lipids, and proteins. The structural organization of the thylakoids includes granal (stacked) lamellae, which contain mainly photosystem II components, and interconnecting stromal (unstacked) lamellae, which preferentially house the photosystem I components and the ATPase complex (1–4). Despite this spatial separation (1, 2), there seems to be a functional cooperation between the two photosystems in regulating light energy distribution and photosynthetic electron transport (4, 5). Indeed, a phosphorylation-dependent shuttling of mobile light-harvesting chlorophyll–protein complex between the photosystems has recently been proposed to mediate this cooperation (4). However, little is known about the molecular events that target newly synthesized components of the photosynthetic complexes to specific loci within the thylakoids. The 32,000-dalton (32-kDa) herbicide-binding protein [also referred to as peak D (6), D1 protein (7), and Q_B protein (8)] is a well-studied integral component of photosystem II (9–11). This rapidly metabolized (6, 12) thylakoid protein is a major light-driven (13) translation product of the chloroplast genome. It contains overlapping binding sites for several classes of herbicides, including the *s*-triazines, ureas, and phenylureas (14). The 32-kDa protein is first synthesized as a 33,500- to 34,500-dalton (33.5-kDa) precursor polypeptide (6, 15, 16). Both the precursor and the mature protein are membrane associated (15, 16). The 32-kDa protein acts at the electron-accepting side of photosystem II (17), possibly as a reaction center component (11, 18, 19).

Circumstantial evidence has recently accumulated suggesting dual membrane locations for the 32-kDa protein. In particular, the discovery of two pools of 32-kDa protein molecules in *Chlamydomonas* (20), the reported absence of the 32-kDa protein from the granal (but not stromal) lamellae in a photosystem II mutant of maize (21), and the demonstration that azidoatrazine binds to a 32-kDa protein only in the granal lamellae (22) raised the possibility that some spatial relocation event was a part of the life cycle of the protein. In this study we demonstrate that the 33.5-kDa precursor protein is exclusively associated with unstacked stromal lamellae. Processing to the mature 32-kDa form occurs in this membrane fraction as well. After processing a translocation of 32-kDa protein to the stacked granal lamellae takes place.

We examined the possibility that posttranslational modification of the 32-kDa protein is involved in its membranal deployment. Reversible covalent modifications such as glycosylation, methylation, phosphorylation, and acylation can have major effects on the activity, conformation, or stability of a protein (23). In plants, glycosylation of seed storage proteins (24) and phosphorylation of chloroplast membrane proteins (4, 25) are well known. Reports on protein acylation (26, 27) however, have been mainly confined to viral glycoproteins (28) and a number of bacterial lipoproteins (29, 30), viral transforming proteins (31–33), and animal membrane proteins (34–37). Acylation of plant proteins has not been reported. Yet, interactions between lipids and protein complexes are known to occur in the thylakoid. In fact, polar lipids and their fatty acid constituents play an important role in the architecture (38, 39) and, probably, function (40) of photosynthetic membranes.

In this study we show that palmitic acid is selectively attached to several chloroplast proteins, including, prominently, the 32-kDa protein. Bound palmitic acid is exclusively associated with the processed 32-kDa form and is readily observed in the stacked granal fraction. At least one palmitoylation site is present in a membrane-protected region of the protein.

MATERIALS AND METHODS

Axenic cultures of *Spirodela oligorrhiza*, an aquatic angiosperm, were used in all experiments. Conditions of growth, *in vivo* radiolabeling, isolation of thylakoids and their fractionation into granal and stromal lamellae, and $NaDodSO_4$/polyacrylamide gel electrophoresis are described in the legends to figures.

RESULTS

Synthesis, Processing, and Translocation of the 32-kDa Protein. Ellis (41) and others (42, 43) have indicated that synthesis of the 32-kDa protein, a core component of photosystem II, occurs on membrane-bound chloroplast ribosomes. As a class, these ribosomes were shown to be enriched in the unstacked stromal lamellae (44). However, photosystem II is known to be concentrated in the stacked granal lamellae (4). This conflict can be conceptually resolved

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Abbreviations: LHCP, light-harvesting chlorophyll *a*/*b* protein complex; LS, large subunit (of ribulose-1,5-bisphosphate carboxylase/oxygenase); ACP, acyl carrier protein.

by postulating spatially distinct sites for 32-kDa protein synthesis and function. Indeed, in recent studies using fractionated lamellae such a model has been assumed (21, 22). We have directly tested this model by following the distribution of newly synthesized 33.5-kDa precursor and 32-kDa processed protein forms in fractionated lamellae.

S. oligorrhiza plants were radiolabeled with [^{35}S]methionine for 3 min, and the radioactivity was "chased" by further incubating the plants in excess nonradioactive methionine. Fig. 1 shows the steady-state (stain) and newly synthesized (fluorogram) polypeptide profiles of stromal and granal lamellae after 0, 6, and 120 min of chase. The stained gel revealed the distinct polypeptide compositions characteristic of granal and stromal lamellae (4, 21): enrichment of photosystem II polypeptides (p47, p43, LHCP) in the granal lamellae, and photosystem I (cpI) and ATPase proteins (α and β) in the stromal lamellae. Electron micrographs showed the stromal lamellar fraction to consist of single-membrane vesicles and the granal lamellar fraction of double membrane sheets. The degree of purity in each fraction, determined by electron microscopy, approached 95% (data not shown).

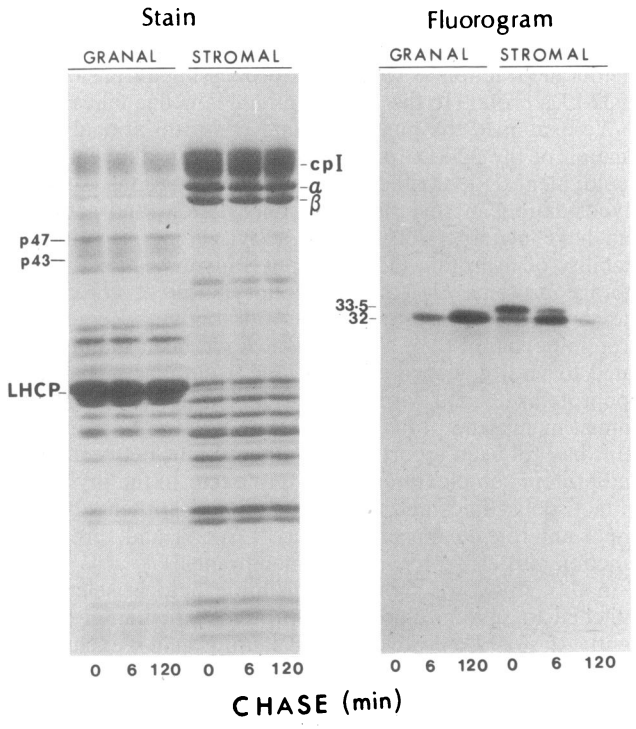

FIG. 1. Intrathylakoid translocation of the 32-kDa protein after its synthesis and processing on stromal lamellae. *S. oligorrhiza* (Kurtz) Hegelm plants were routinely grown phototrophically (25°C, 20 μmol·m^{-2}·s^{-1} photosynthetically active radiation) under cool white fluorescent lamps for 10–15 days as axenic cultures on half-strength Hutner's mineral medium (45) containing 0.5% sucrose. For pulse labeling, these cultures were transferred for 2–3 days to mineral medium lacking sucrose. The plants were labeled in the light for 3 min with 660 μCi (1 Ci = 37 GBq) of [^{35}S]methionine in 1 ml of mineral medium and then washed immediately in nonradioactive mineral medium containing 1 mM methionine (chase medium). Samples were removed after 0, 6, and 120 min of incubation in chase medium and each was mixed with 2–3 g (fresh weight) of unlabeled plants. Whole thylakoids were isolated and fractionated into granal and stromal lamellae as described by Leto *et al.* (21). Polypeptides associated with these membranes were analyzed on NaDodSO$_4$/10–20% polyacrylamide gradient gels as previously described (17). The gels were stained with Coomassie blue R (0.2% in 50% methanol/7% acetic acid/43% water, vol/vol), destained in 20% methanol/7% acetic acid/73% water (46), fluorographed using EN-^{3}HANCE (New England Nuclear), dried, and exposed on Curix RP2 x-ray films (Agfa). The positions of the photosystem I reaction center polypeptide (cpI), α and β subunits of proton ATPase (α, β), photosystem II polypeptides [p47, p43, and light-harvesting chlorophyll *a*/*b* protein complex (LHCP)] are indicated on the stained gel, while the positions of the radiolabeled 33.5-kDa precursor polypeptide (33.5) and the processed 32-kDa protein (32) are indicated on the fluorogram.

The fluorogram in Fig. 1 shows that after 3 min of labeling (i.e., 0-time chase) virtually all of the radioactivity in the membrane was in the stromal lamellar fraction, with the 33.5-kDa precursor polypeptide being the most prominent band. Processing of the precursor polypeptide to the 32-kDa protein also occurred in the stromal lamellae (6-min chase). However, by 120 min little radioactivity remained in this fraction. On the other hand, accumulation of radioactivity in the granal lamellae commenced only after 6 min of chase, with the processed 32-kDa protein being the only prominent band. By 120 min of chase virtually all of the radioactivity associated with the 3-min-pulsed plants was concentrated in the 32-kDa protein band of the granal lamellae. These data demonstrate that membrane attachment and processing of the 33.5-kDa precursor polypeptide occur in the stromal lamellae. However, once processed, the 32-kDa protein is translocated to the topologically distinct granal lamellae.

Posttranslational Palmitoylation of Chloroplast Proteins. Posttranslational acylation of viral and mammalian proteins has variously been implicated in mediating polypeptide transport and processing, anchoring of proteins in the membrane, and assembly of protein complexes (26, 27). We took note of the high lipid content of the chloroplast and sought to determine if an acylation signal was involved in regulating some aspect of 32-kDa protein organization within the membrane. To this end, *Spirodela* plants were pulse-labeled in the light for 3 min with [^{3}H]palmitic acid, the major saturated fatty acid species in the plastid. Membrane and soluble fractions were then prepared and analyzed by NaDodSO$_4$/PAGE. Fig. 2 shows the distribution of the ^{3}H-labeled and steady-state (stained) protein bands. Two prominent radioactive bands were seen in the membrane fraction. One electrophoresed with [^{35}S]methionine-labeled 32-kDa protein and the other with the high-abundance LHCP. Some of the radioactivity associated with the membrane fraction migrated near the gel front. Two prominent radioactive bands were also seen in the soluble fraction. One electrophoresed with the large subunit (LS) of ribulose-1,5-bisphosphate carboxylase/oxygenase, and the other showed a molecular mass of 10 kDa, similar to that of acyl carrier protein (ACP) (47). The identities of three of the major bands were verified in further experiments: trypsinization of isolated ^{3}H-labeled membranes revealed the characteristic pattern (46) for the 32-kDa protein (see Fig. 5); antibodies raised against ribulose-1,5-bisphosphate carboxylase/oxygenase (48) specifically precipitated a ^{3}H-labeled polypeptide electrophoresing with authentic LS (data not shown), while antibodies raised against spinach ACP (47) specifically precipitated a ^{3}H-labeled polypeptide having an electrophoretic mobility corresponding to 10 kDa and electrophoresing with authentic acyl-ACP (A.K.M., F. E. Callahan, R. A. Mehta, and J. B. Ohlrogge, unpublished data).

Fig. 3 compares *in vivo* labeling of proteins with [^{3}H]palmitic acid in the light and in darkness. The uptake of [^{3}H]palmitate by *Spirodela* fronds during 1 min of incubation in the light was similar to that in the dark (1.6×10^8 versus 1.2×10^8 cpm/mg of chlorophyll, respectively). However, the intensities of the four prominent ^{3}H-labeled protein bands were considerably greater in the light-incubated samples. In analyzing these data we took note of the fact that light also stimulates synthesis of several of these proteins (12, 13). However, at least for the 32-kDa protein, any increase in the amount of protein due to synthesis in the light, during the 1-min labeling period, would be unlikely to account for stimulation of protein acylation. This is because palmitoyla-

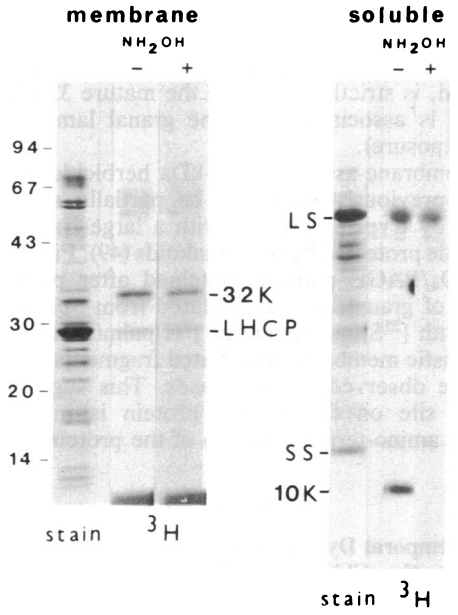

FIG. 2. *In vivo* labeling of *Spirodela* proteins with [^{3}H]palmitic acid. Plants were grown in the light as described in the legend to Fig. 1. The plants were radiolabeled in the light (30 μmol·m^{-2}·s^{-1} photosynthetically active radiation, 25°C) for 3 min with 700–1000 μCi of [9,10-^{3}H(N)]palmitic acid (15.2 Ci/mmol; New England Nuclear) in 1 ml of mineral medium. Prior to use, radioactive palmitic acid solution was dried under a stream of nitrogen gas and then dissolved in dimethyl sulfoxide. The concentration of dimethyl sulfoxide in the labeling medium was kept under 1%. After labeling, the plants were washed twice with 5–10 ml of mineral medium and immediately frozen on dry ice until used. Cell homogenization and fractionation into soluble and membrane proteins were carried out as previously described (16). Samples for gel electrophoresis were mixed with application buffer (17), fractionated by NaDodSO$_4$/PAGE, and stained with Coomassie blue as described in the legend to Fig. 1. On the left, molecular masses in kDa are indicated. The gels were then destained in 25% isopropyl alcohol/10% acetic acid/65% water (vol/vol) (31), fluorographed using EN^{3}HANCE, dried, and exposed on Curix RP2 x-ray films. The stability of the acyl linkage of [^{3}H]palmitic acid was tested as follows. Duplicate gels were run. After fixation, one replicate was washed in water to remove acetic acid and incubated for 24 hr at 25°C with 1 M NH_2OH·HCl (pH 6.6) (31). The second gel was incubated without NH_2OH·HCl. Both gels were then dried and fluorographed. The positions of the large subunit (LS) and the small subunit (SS) of ribulose-1,5-bisphosphate carboxylase/oxygenase, the 10-kDa ACP (10K), the LHCP, and the 32-kDa herbicide-binding protein (32K) are indicated.

tion occurs at least 3–6 min after synthesis of the 33.5-kDa protein (cf. Fig. 4). Thus, the association of palmitic acid with these proteins is a bona fide light-stimulated event.

The rapidity with which *Spirodela* proteins become labeled with [^{3}H]palmitate in the light (1–3 min) minimized the risk of metabolism of the labeled moiety. Nonetheless, we carried out experiments to directly identify the radiolabel in the protein bands after PAGE. The migrations of methyl palmitate, authentic [^{3}H]palmitic acid, and the ^{3}H-labeled material extracted from the putative LHCP gel band hydrolyzed in methanolic HCl were compared. The chromatographic migration of the major peak of radioactivity from the gel band matched the migrations of the palmitate standards (data not shown). In other experiments, using high-pressure liquid chromatographic analysis of the ^{3}H-labeled material extracted from the 32-kDa protein gel band, the labeled moiety was identified as palmitic acid. No label was detected as myristic acid. These data indicate that the ^{3}H-labeled moiety in the rapidly labeled protein bands remains as palmitic acid. Finally, some plants were incubated for 1–3 min with

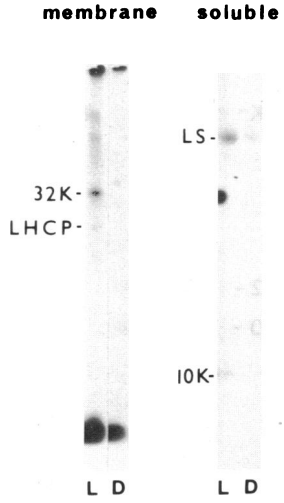

FIG. 3. Light-stimulated [^{3}H]palmitic acid labeling of chloroplast proteins. Phototrophically grown *Spirodela* plants were labeled for 1 min in the dark (D) or in the light at 30 μmol·m^{-2}·s^{-1} photosynthetically active radiation (L) with [^{3}H]palmitic acid, washed with 5 ml of mineral medium, and immediately frozen on dry ice. Soluble and membrane fractions were isolated and analyzed by NaDodSO$_4$/PAGE as described in the legend to Fig. 2. The positions of prominent [^{3}H]palmitic acid-labeled proteins are indicated (abbreviations as in Fig. 2).

[^{3}H]palmitic acid, [^{3}H]myristic acid, or [^{3}H]glycerol, and membrane proteins were fractionated by NaDodSO$_4$/PAGE. Although a few proteins were labeled with [^{3}H]myristic acid, the intensity of label was minimal compared to that seen with [^{3}H]palmitic acid (F. E. Callahan, H. Norman, J. B. St. John, and A.K.M., unpublished data). No radioactive protein bands were observed in samples incubated with [^{3}H]glycerol. These results suggest that palmitic acid is a major fatty acid ligand for acylation of proteins in the chloroplast.

The type of acyl linkage to the chloroplast proteins was investigated by incubating NaDodSO$_4$/polyacrylamide gels in 1 M NH_2OH at pH 6.6. As seen in Fig. 2, the [^{3}H]palmitate-labeled 10-kDa protein band was sensitive to acidic NH_2OH, indicating a thiol ester linkage. The remaining ^{3}H-labeled

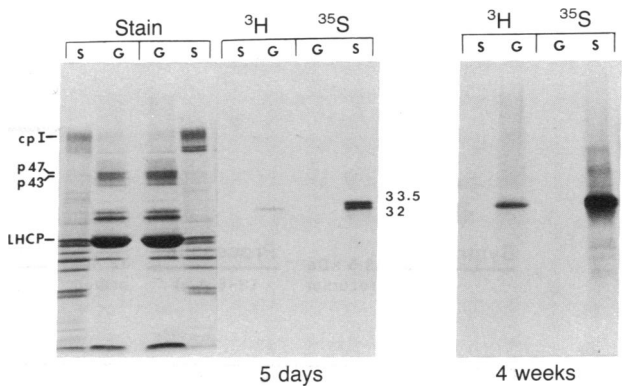

FIG. 4. [^{3}H]Palmitic-acid-labeled 32-kDa protein is associated with granal lamellae. *Spirodela* plants were radiolabeled either with [^{3}H]palmitic acid for 3 min or with [^{35}S]methionine for 6 min in the light as described in the legends to Figs. 1 and 2. Isolation and fractionation of granal (G) and stromal (S) lamellae were as described by Leto *et al.* (21). NaDodSO$_4$/PAGE, staining, and fluorography were as described in the legend to Fig. 1. The positions of the 33.5-kDa precursor polypeptide (33.5) and 32-kDa herbicide binding protein (32) in the [^{3}H]palmitate- and [^{35}S]methionine-labeled gel lanes are indicated. Short (5 days) and longer (4 weeks) exposures of the gel are shown. The positions of marker polypeptides (cf. legend to Fig. 1) are indicated.

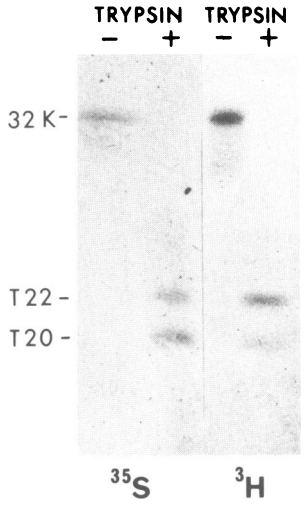

FIG. 5. [3H]Palmitic acid labeling of the 32-kDa protein occurs in the trypsin-resistant amino-terminal region. *Spirodela* plants were labeled in the light either with [3H]palmitic acid for 3 min, as described in the legend to Fig. 2, or with 660 μCi of [35S]methionine in 1 ml of mineral medium for 60 min. Granal lamellae were isolated (21) and incubated with or without trypsin (1 μg of enzyme per μg of chlorophyll equivalent of membrane) for 30 min as previously described (46). The two characteristic membrane-associated tryptic fragments (T22 and T20) of the 32-kDa protein (32 K) (46) are evident in both the 35S- and 3H-labeled samples.

bands were resistant to this treatment, suggesting a stronger linkage such as ether or amide (35).

Palmitoylation of the 32-kDa Protein in Relation to Its Translocation. Palmitoylation of the 32-kDa herbicide-binding protein was studied in further detail. *Spirodela* plants were labeled with either [35S]methionine for 6 min or [3H]palmitate for 3 min, and granal and stromal lamellae were isolated from whole thylakoids. Fig. 4 shows the distinctive, stained protein pattern obtained after NaDodSO4/PAGE for each lamellar type, as well as two fluorographic exposures of the gel. In agreement with the results shown in Fig. 1, the distribution of the methionine label indicates that synthesis and processing of the 33.5-kDa precursor polypeptide occur exclusively at the stromal lamellae. Palmitoylation, on the other hand, is strictly limited to the mature 32-kDa protein band and is associated with the granal lamellae (cf. the 4-week exposure).

The membrane-associated 32-kDa herbicide-binding protein was previously shown to be partially susceptible to digestion by trypsin (15, 17), with a large fragment of the polypeptide protected by the thylakoids (49). Fig. 5 shows the NaDodSO4/PAGE patterns obtained after partial trypsin digestion of granal lamellae isolated from *Spirodela* plants labeled with [35S]methionine or [3H]palmitic acid. The two characteristic membrane-associated fragments, T22 and T20 (46), were observed in both cases. This suggests that an acylation site on the 32-kDa protein is in the trypsin-protected amino-terminal region of the protein.

DISCUSSION

Spatiotemporal Dynamics of the 32-kDa Herbicide-Binding Protein in the Chloroplast Membranes. Several features concerning the 32-kDa herbicide-binding protein have been delineated in this report. These include the subcellular site of processing, membrane translocation, and posttranslational acylation. Interrelationships among these and other dynamic membrane events associated with the metabolism of the 32-kDa protein are outlined in Fig. 6. A pivotal occurrence is intrathylakoidal translocation. This is an early event in the life span of the protein and results in a physical separation between the site of precursor processing and the likely major site of 32-kDa protein function. Interestingly, a 30-kDa polypeptide, most probably the D2 protein, another photosystem II core protein (11, 19, 52), likewise undergoes translocation from stromal to granal lamellae (56). Perhaps relocation within the thylakoid is a general feature of chloroplast-encoded photosystem II proteins.

Superimposed on these movements is the palmitoylation of the 32-kDa protein (Fig. 6). It is tempting to suggest that palmitoylation of the processed protein may promote its proper functional integration in the photosystem II complex

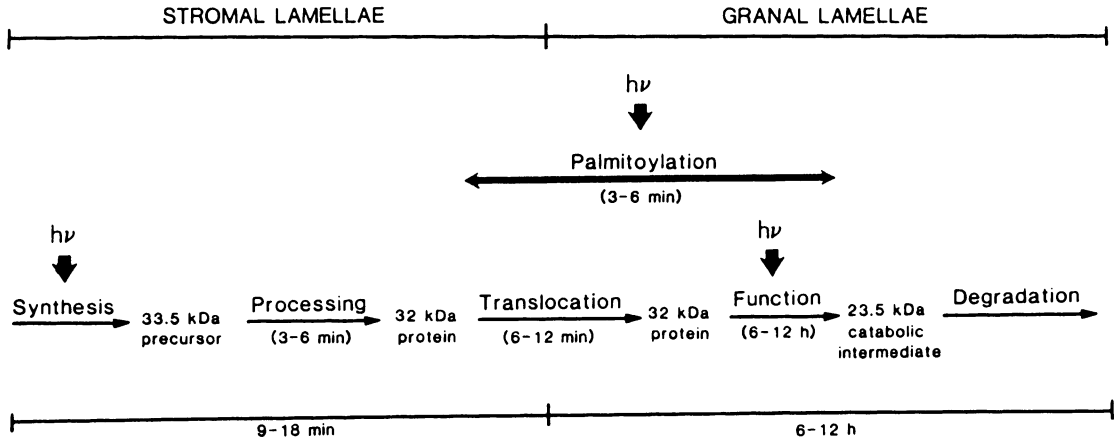

FIG. 6. Membrane-associated events in the life of the 32-kDa herbicide-binding protein. All time values represent estimated half-times for the protein in *Spirodela* cultured at 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation. Light-dependent synthesis (12, 13) of nascent 33.5-kDa precursor polypeptide takes place on 70S thylakoid-bound ribosomes (41–43), most likely in the unstacked stromal lamellae (44). After completion of translation, the 33.5-kDa polypeptide is associated exclusively with the stromal lamellae (Figs. 1 and 4). At this location, carboxyl-terminal processing (49) occurs with a half-time of 3–6 min (16) (Figs. 1 and 4). After processing, the 32-kDa protein is translocated within the thylakoids to the stacked granal lamellae with a half-time of 6–12 min (Fig. 1). At this new location, where photosystem II is primarily concentrated (4, 21), the 32-kDa protein is thought to be functionally active and gains the ability to bind herbicides (22). The half-time of the 32-kDa protein, which is thought to normally remain in the granal lamellae, is 6–12 hr (50). Thus, the protein spends the majority of its life span in the granal lamellae. Light-intensity-dependent degradation of the 32-kDa protein (12) occurs in the granal lamellae, with production of a 23.5-kDa membrane-bound catabolic intermediate (51). At some stage(s) between 32-kDa protein translocation and formation of the catabolic intermediate, light-induced (Fig. 3) palmitoylation of the amino-terminal region of the protein occurs (Figs. 4 and 5). This tight binding (Fig. 2) of fatty acid to the 32-kDa protein is a short-lived event (half-time about 3–6 min) (56).

(53) once translocation has occurred. Experiments using inhibitors of acylation and of translocation can be used to test this idea. We have also recently determined that palmitoylation of the 32-kDa protein is a relatively short-lived event, with deacylation occurring rapidly in the granal lamellae (F. E. Callahan, A.K.M., and M.E., unpublished data). Nonetheless, present data do not permit assigning this acylation to a precise temporal or even spatial stage in the life cycle of the protein. For example, while the data in Fig. 4 clearly fail to reveal palmitoylated protein in the stromal lamellae, we cannot rule out its ephemeral existence at this location. In such a case, protein modification might serve primarily as a tag leading to rapid translocation of the 32-kDa protein to the grana.

The other three chloroplast proteins identified here as undergoing palmitoylation in intact plants are, like the 32-kDa protein, matured forms of higher-molecular-weight precursor polypeptides (47, 54, 55). This fact tends to preclude a role for protein palmitoylation in the transport of the nucleus-encoded LHCP or ACP precursor polypeptides to the chloroplast or in their subsequent processing. Likewise, an involvement in the processing of the plastid-encoded LS protein seems unlikely. The physiological role of palmitoylation in all these cases awaits further study.

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